P1 127637

EJ

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

August 30, 1999

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/088,568*
FILING DATE: *June 09, 1998*
PCT APPLICATION NUMBER: *PCT/US99/12900*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

R. BLAKENEY
Certifying Officer

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Aprov

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (b)(2).

Docket Number	UCON/140/US	Type a plus sign (+) inside this box -->	+

INVENTOR(s)/APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
Makriyannis	Alexandros		Watertown, MA, USA
Lin	Sonyuan		Storrs, CT, USA

TITLE OF THE INVENTION (280 characters max)

Anandamide Transporter Inhibitor Medications

CORRESPONDENCE ADDRESS

James E. Alix, Esq.
Alix, Yale & Ristas, LLP
750 Main Street
Hartford

CUSTOMER NO. 002543

STATE	CT	ZIP CODE	06103-2721	COUNTRY	U S.A.

ENCLOSED APPLICATION PARTS (check all that apply)

X Specification — Number of Pages 8
X Small Entity Statement
__ Drawing(s) — Number of Pages __
__ Other (specify) __

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)

X A check or money order is enclosed to cover the filing fees
X The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number: 16-2563

FILING FEE AMOUNT ($): $ 150

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government

X No
__ Yes, the name of the U S. Government agency and the Government contract number are: __

Respectfully submitted,

SIGNATURE James E. Alix Date June 9, 1998

TYPED or PRINTED NAME James E. Alix REGISTRATION NO. 20,736

__ Additional inventors are being named on separately numbered sheets attached hereto.

EXPRESS MAIL mailing label number EL 052 086 112 US

Date of Deposit June 9, 1998

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D C 20231.

Rose A. Smollen

Rose A. Smollen

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, DC 20231.

Anandamide Transporter Inhibitor Medications

INVENTION DISCLOSURE Page 1 Disclosure No._____

(1) COMPLETE DESCRIPTION OF THE INVENTION: Use additional pages, if necessary, and attach any relevant sketches, diagrams, drawings, photographs or other illustrative material. ALL ATTACHED MATERIALS MUST BE SIGNED AND DATED BY EACH INVENTOR AND WITNESSED. Description may be by reference to a separate document such as a publication, manuscript, preprint or report. Such documents must be attached.

A carrier protein that transports extracellular anandamide across the cell membrane has been shown to be present in rat neurons and astrocytes. This carrier protein or anandamide transporter is believed to be responsible for the inactivation of anandamide, an endogenous cannabinoid for central cannabinoid receptors. Thus, anandamide released from neurons on depolarization is rapidly transported back into the cells and subsequently hydrolyzed by an amidase thereby terminating its biological actions. Anandamide transporter is a potential therapeutic target for the development of useful medications.

We have discovered a phenolic analog of anandamide namely N-(4-hydroxyphenyl)arachidonylamide (AM404) which inhibits the transport of anandamide across the cell membranes. AM404 does not activate cannabinoid receptors or inhibit anandamide hydrolysis *per se*. However, it does potentiate receptor-mediated anandamide responses by preventing anandamide reuptake. .....Continued on Supplement Page

(2) NOVEL FEATURES: Clearly specify the novel aspects of your invention. Compared to present technology, how is your invention different?

AM404 is a potent inhibitor of anandamide transport and it is the only compound known todate that competitively inhibits anandamide reuptake.

What deficiency in the present technology does your invention improve upon? Is it more effective? cheaper? superior in other ways?

Present cannabinoid drugs are targeted towards cannabinoid receptors (CB1 and CB2) and anandamide amidase enzyme. AM404 described in this invention targets a novel protein called anandamide transporter.

(3) STAGE OF DEVELOPMENT: Cite your specific results to date demonstrating that your concept is valid. Has your work included laboratory studies? Pilot-scale experiments? Construction and testing of a prototype?

AM404 inhibited accumulation of anandamide in rat neurons and astrocytes with an IC_{50} of 1μM for neurons and 5μM for astrocytes. In addition, AM404 potentiated and prolonged receptor-mediated effects of anandamide such as vasodialation. These experiments further support that AM404 is an inhibitor of anandamide transport.

Inventor(s) 1. A Makriyannis Date 5/12/98 Disclosed to and Understood by:

2. Sony Gaonkar Date 5/12/98 A Makriyannis Date 5/12/98

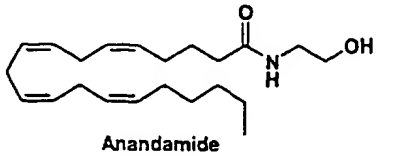
3. _____ Date _____ Vishwanath Date 5/12/98

SUPPLEMENT PAGE

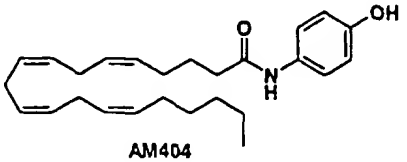
INVENTION DISCLOSURE FORM Disclosure No. _____

Continued from page 1, item 1:

Structural formulas for AM404 and anandamide are shown below.



Anandamide



AM404

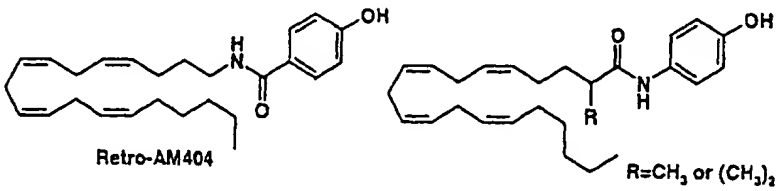
AM404 and its analogs are potential drug candidates for the treatment of ailments related to the cannabinoid system. Potential therapeutic uses of AM404 are pain alleviation (analgesia), treatment of cardiovascular diseases and blood pressure disorders.

Inventor(s) 1. A. Makriyannis Date 5/12/98 Disclosed to and Understood by:

2. Song Cheng Date 5/12/98 Date 5/12/98

3. _____ Date _____ Date 5/12/98

(4) VARIATIONS OF THE INVENTION: Discuss all alternate forms that you can foresee for this invention, whether or not you have evaluated them to date. (For example, chemical inventions should consider analogs and derivatives.)



AM404 was first synthesized in March 1993 and tested in July 1997 as anandamide transport inhibitor

(6) INVENTOR'S PUBLICATION PLANS: Please list all your publications — theses, reports, pre-prints, abstracts, papers, etc. that pertain to the invention. Include publication dates. Also, include manuscripts for publication (submitted or not), news releases, and internal publications. Enclose copies of all the above items with this disclosure.

Beltramo, M.; Stella, N.; Calignano, A.; Lin, S.; Makriyannis, A.; Piomelli, D. Functional Role of High Affinity Anandmide Transport Inhibitor, as Revealed by Selective Inhibition. Science 1997, 277, 1094. (included) - CD8

BioWorld Today, Volume 8(162), August 21, 1997.

(7) PRIOR DISCLOSURE: Please give the details (date, place and circumstances) of any oral or written disclosures of all or part of this invention. If disclosed to specific individuals, give their names. Include professional meetings and conferences. Has this invention or a product resulting from this invention been offered for sale or license? Have any samples related to this invention been distributed?

No prior disclosure

Inventor(s) 1. A. Makriyannis Date 5/12/98 Disclosed to and Understood by:

2. Steve Lin Date 5/12/98 A. Makriyannis Date 5/12/98

3. _____ Date _____ Mahavir Khanna Date 5/12/98

SUPPORTING INFORMATION

(1) PRIOR KNOWLEDGE AND COMPETING RESEARCH AND DEVELOPMENT: Please list all publications and patents by the inventor or others that relate to the invention. The inventor should thoroughly search the published literature and review closely related patents. Publications by the researchers:

1) Calignano, A.; La Rana, G.; Beltramo, M.; Makriyannis, A.; Piomelli, D. Potentiation of Anandamide Hypotension by the Transport Inhibitor, AM404. *Eur. J. Pharmacol.* 1997, *337*, R1-R2. 2) Calignano, A.; La Rana, G.; Makriyannis, A.; Lin, S.; Beltramo, M.; Piomelli, D. Inhibition of Intestinal Motility by Anandamide, an Endogenous Cannabinoid. *Eur. J. Pharmacol.* 1997, *340*, R7-R8.

List any known research groups currently engaged in research and development in this area. Include both academic and industrial researchers.

None

(2)ALTERNATE TECHNOLOGY: Describe any known alternate technologies that accomplish the same or similar purposes as this invention. List companies and products that currently use these alternate technologies.

None

(3) COMMERCIAL APPLICATION OF THE INVENTION: List all products, processes, devices, equipment, etc., to which your invention could be applied or which could result directly from your invention. Can these applications be developed in the near term (within two years) or the long term (more than two years)?

Medication to alleviate pain and treatment of cardiovascular diseases.
Long term development

What firms or types of firms do you think may be interested in the invention? Why? Name companies and specific persons if possible. Especially list companies with which you have had direct contact.

Pharmaceutical and biotech companies

(4) RESEARCH AND DEVELOPMENT PLANS: What additional research is needed to complete development and testing of the invention? Are you actively pursuing the needed work? Under whose sponsorship? About how long will this work take? What additional research support, if any, is needed for these efforts?

IDENTIFICATION AND FUNCTIONAL ROLE OF HIGH AFFINITY ANANDAMIDE TRANSPORT

M. Beltramo*, N. Stella*, A. Calignano#, S.Y. Lin+, A. Makriyannis+ and D. Piomelli*. *The Neurosci. Inst. San Diego, CA 92121, #Sch.of Pharm. Univ. of Naples, Italy 80131, and +Sch. of Pharm. Univ. of Connecticut, Storrs, CT 06269.

Anandamide (arachidonylethanolamide) is an endogenous lipid that activates brain cannabinoid receptors. Two main pathways have been proposed for anandamide inactivation: cellular uptake and enzymatic degradation. In the present study we identified and characterized pharmacologically a high affinity anandamide uptake system in neurons and astrocytes. Exogenous [^{3}H]anandamide (spec. rad.: 221 Ci/mmol) is rapidly cleared ($t_{1/2}$=4 minutes) from the media of neurons or astrocytes in cell culture through a saturable, temperature-dependent and sodium-independent transport system. This uptake displays high affinity for [^{3}H]anandamide (neurons: Km 1.2 microM; astrocytes: Km 0.32 microM). Competition experiments with fatty acid derivatives, arachidonic acid, or palmitoylethanolamide proved its specificity. Screening of lipid uptake blockers and anandamide analogs led to the identification of a compound N-(4-hydroxyphenyl) arachidonylamide (AM404) which is potent and specific in inhibiting anandamide transport, but does not activate CB1 cannabinoid receptors and does not inhibit anandamide degradation. In cultures of cortical neurons, concentrations of anandamide higher than 0.3 microM are necessary to activate CB1 cannabinoid receptors and to revert forskolin-induced adenylyl cyclase activity. In the presence of AM404 (10 microM) the potency of anandamide is greatly increased. By contrast, AM404 has no effect on adenylyl cyclase activity when applied alone (10 microM), and does not potentiate adenylyl cyclase activity inhibition elicited by the CB1 receptor agonist WIN-55212-2 (100 nM) or by glutamate (3 microM). The hot-plate model of analgesia in the mouse was used to test the functional role of anandamide transport *in vivo*. Intravenous (i.v) administration of anandamide (20 mg/kg) induces a modest, but significant, analgesia which disappears 60 minutes after the injection and is prevented by SR-141716 (1 mg/kg, i.p.). Administration of AM404 (10 mg/kg, i.v.) has no antinociceptive effect *per se* within 60 minutes of injection, but significantly enhances and prolongs anandamide-induced analgesia. The identification in neural cells of a high-affinity [^{3}H]anandamide transport system and the discovery of selective transport blockers should be important to understand the physiological role of the endogenous cannabinoid system. In light of the multiple behavioral effects of cannabinoid receptor activation, these inhibitors might also open novel therapeutic avenues for the treatment of psychiatric and neurological disorders.

Work at The Neurosciences Institute was supported by Neurosciences Research Foundation which receives major support from Sandoz Pharm. S.Y. Lin and A. Makriyannis were supported by a grant (DA-3801) from NIDA.

Functional Role of High-Affinity Anandamide Transport, as Revealed by Selective Inhibition

M. Beltramo, N. Stella, A. Calignano, S. Y. Lin, A. Makriyannis, D. Piomelli*

Anandamide, an endogenous ligand for central cannabinoid receptors, is released from neurons on depolarization and rapidly inactivated. Anandamide inactivation is not completely understood, but it may occur by transport into cells or by enzymatic hydrolysis. The compound *N*-(4-hydroxyphenyl)arachidonylamide (AM404) was shown to inhibit high-affinity anandamide accumulation in rat neurons and astrocytes in vitro, an indication that this accumulation resulted from carrier-mediated transport. Although AM404 did not activate cannabinoid receptors or inhibit anandamide hydrolysis, it enhanced receptor-mediated anandamide responses in vitro and in vivo. The data indicate that carrier-mediated transport may be essential for termination of the biological effects of anandamide, and may represent a potential drug target.

Anandamide (arachidonylethanolamide) is an endogenous lipid that activates brain cannabinoid receptors and mimics the pharmacological effects of Δ^9-tetrahydrocannabinol, the active principle of hashish and marijuana (*1*). In humans, such effects include euphoria, calmness, dream states, and drowsiness (*2*). Depolarized neurons release anandamide (*3*) through a mechanism that may require the calcium-dependent cleavage of a phospholipid precursor in neuronal membranes (*4*). Like other modulatory substances, extracellular anandamide is thought to be rapidly inactivated, but the exact nature of this inactivating process is still unclear. A possible pathway is hydrolysis to arachidonic acid and ethanolamine, catalyzed by a membrane-bound fatty acid amide hydrolase (FAAH) highly expressed in rat brain and liver (*5*). Nevertheless, the low FAAH activity found in brain plasma membranes indicates that this enzyme may be intracellular (*5*), a possibility that is further supported by sequence analysis of rat FAAH (*6*). Although anandamide could gain access to FAAH by passive diffusion, the transfer rate is expected to be low because of the molecular size of this lipid mediator (*7*). In that other lipids including polyunsaturated fatty acids and prostaglandin E_2 (PGE_2) enter cells by carrier-mediated transport (*8, 9*), it is possible that anandamide uses a similar mechanism. Indeed, the existence of a rapid, saturable process of anandamide accumulation into neural cells has been reported (*3*). This accumulation may result from the activity of a transmembrane carrier, which may thus participate in termination of the biological actions of anandamide. Accordingly, we developed drug inhibitors of anandamide transport and investigated their pharmacological properties in cultures of rat cortical neurons or astrocytes.

The accumulation of exogenous [^{3}H]anandamide by neurons or astrocytes fulfills several criteria of a carrier-mediated transport (Fig. 1) (*10*). It is a rapid process that reaches 50% of its maximum within about 4 min (Fig. 1A). Furthermore, [^{3}H]anandamide accumulation is temperature-dependent (Fig 1A) and saturable (Fig. 1, B and C). Kinetic analyses revealed that accumulation in neurons can be represented by two components of differing affinities (lower affinity: Michaelis constant, K_m, = 1 2 μM, maximum accumulation rate, V_{max}, = 90.9 pmol/min per milligram of protein; higher affinity: K_m = 0.032 μM, V_{max} = 5 9 pmol/min per milligram of protein) (Fig. 1B). The higher affinity component may reflect a binding site, however, as it is displaced by the cannabinoid receptor antagonist, SR-141716-A (100 nM) (*11*). In astrocytes, [^{3}H]anandamide accumulation is represented by a single high-affinity component (K_m = 0.32 μM, V_{max} = 171 pmol/min per milligram of protein) (Fig. 1C). Such apparent K_m values are similar to those of known neurotransmitter uptake systems (*12*) and are suggestive therefore of high-affinity carrier-mediated transport.

To characterize further this putative anandamide transport, we used cortical astrocytes in culture As expected from a selective process, the temperature-sensitive component of [^{3}H]anandamide accumulation was prevented by nonradioactive anandamide, but not by palmitoylethanolamide, arachidonate, prostanoids, or leukotrienes (Fig. 2A). Replacement of extracellular Na^+ with *N*-dimethylglucosamine or choline had no effect (as percentage of control: *N*-dimethylglucosamine, 124 ± 12%; choline, 98 ± 14%; mean ± SEM, n = 6), suggesting that [^{3}H]anandamide accumulation is mediated by a Na^+-independent mechanism, which has been observed with other lipids (*8, 9*). Moreover, inhibition of FAAH activity by treating the cells with (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (25 μM) or linoleyl trifluoromethyl ketone (15 μM) (*13, 14*) had no effect (Fig. 2, B and C) This indicates that anandamide hydrolysis did not provide the driving force for anandamide transport into astrocytes within the

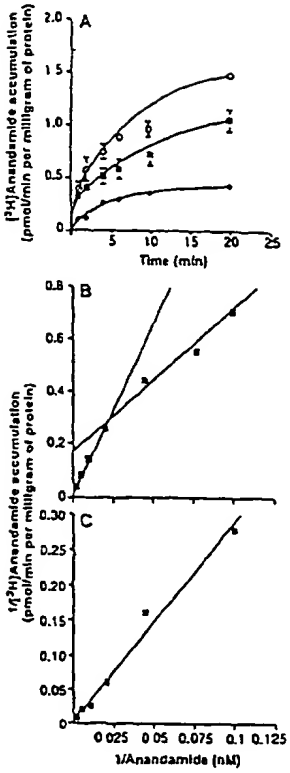


Fig. 1. (A) Time course of [^{3}H]anandamide accumulation in rat cortical neurons (circles) or astrocytes (squares) at 37°C, and astrocytes at 0° to 4°C (diamonds). Results are expressed as mean ± SEM of 6 to 12 independent determinations. (B and C) Lineweaver-Burk analyses of [^{3}H]anandamide accumulation (37°C, 4 min) in neurons (B) or astrocytes (C). Results are from one experiment representative of three performed in duplicate with each cell type. The [^{3}H]anandamide accumulation assay has been described (*10*).

M. Beltramo, N. Stella, D. Piomelli, The Neurosciences Institute, 10640 J. J. Hopkins Drive, San Diego, CA 92121, USA.
A. Calignano, School of Pharmacy, University of Naples, Italy 80131.
S. Y. Lin and A. Makriyannis, School of Pharmacy, University of Connecticut, Storrs, CT 06269, USA.
*To whom correspondence should be addressed. E-mail: piomelli@nsi.edu

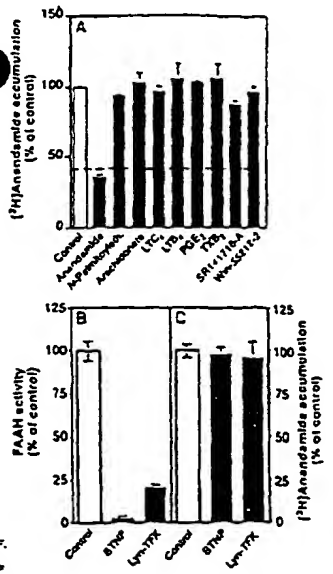


Fig. 2. (A) Selectivity of [³H]anandamide accumulation in cortical astrocytes. Accumulation was measured after a 4-min incubation with [³H]anandamide at 37°C, in the absence (control) or presence of nonradioactive anandamide (100 μM), *N*-palmitoylethanolamide (100 μM), arachidonate (100 μM), leukotriene C_4 (LTC_4; 1 μM), leukotriene B_4 (LTB_4; 1 μM), PGE_2 (100 μM), or thromboxane B_2 (TXB_2; 50 μM). The broken line indicates nonspecific [³H]anandamide accumulation in cells measured at 0° to 4°C (43 ± 3% of total accumulation, which in these experiments was 43,104 ± 1249 dpm per well). Results are expressed as mean ± SEM (n = 6 to 9). Effects of FAAH inhibitors on (B) FAAH activity and (C) [³H]anandamide accumulation in cortical astrocytes. Cells were incubated for 10 min with (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (BTNP, 25 μM) or linoleyl trifluoro methylketone (Lyn-TFK, 15 μM), and then with the same drugs plus [³H]anandamide for an additional 20 min. The total radioactivity in cell lipid extracts (to measure [³H]anandamide transport) (*10*) and radioactivity in nonesterified arachidonate (to measure FAAH activity) (*13*) were measured separately in samples of lipid extracts prepared from the same cultures.

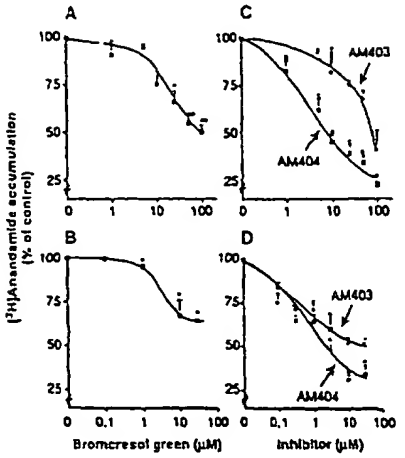


Fig. 3. Inhibition of [³H]anandamide accumulation by bromcresol green in (A) astrocytes or (B) neurons. One asterisk indicates $P < 0.05$ and two asterisks $P < 0.01$ [analysis of variance (ANOVA) followed by Bonferroni test] compared with control [³H]anandamide accumulation. Inhibition of [³H]-anandamide accumulation by AM404 (squares) or AM403 (diamonds) in (C) astrocytes or (D) neurons. The asterisk indicates $P < 0.05$ (paired Student's t test between AM404 and AM403). In all experiments, cells were incubated with the inhibitors for 10 min before the addition of [³H]anandamide for an additional 4 min. Results are expressed as mean ± SEM of three to nine independent determinations.

time frame of our experiments. Finally, the cannabinoid receptor agonist WIN-55212-2 (1 μM) and antagonist SR-141716-A (10 μM) also had no effect, suggesting that receptor internalization was not involved (Fig. 2A).

A primary criterion for defining carrier-mediated transport is pharmacological inhibition. To identify inhibitors of anandamide transport, we first examined compounds that prevent the cellular uptake of other lipids, such as fatty acids (phloretin, 50 μM), phospholipids (verapamil, 100 μM; quinidine, 50 μM), or PGE_2 (bromcresol green, 0.1 to 100 μM) (*15*). Among the compounds tested, only bromcresol green interfered with anandamide transport, albeit with limited potency and partial efficacy (Fig. 3, A and B). Bromcresol green inhibited [³H]anandamide accumulation with an IC_{50} (concentration needed to produce half-maximal inhibition) of 4 μM in neurons and 12 μM in astrocytes and acted noncompetitively (*16*). Moreover, bromcresol green had no significant effect on the binding of [³H]WIN-55212-2 to rat cerebellar membranes (inhibition constant, K_i = 22 μM), FAAH activity in rat brain microsomes (IC_{50} > 50 μM), and uptake of [³H]arachidonate or [³H]ethanolamine in astrocytes (121 ± 13% and 103 ± 12%, respectively, at 50 μM bromcresol green, n = 3) (*17*). The sensitivity to bromcresol green, which blocks PGE_2 transport, raised the question of whether anandamide accumulation occurred by means of a PGE_2 carrier. That this is not the case was shown by the lack of [³H]PGE_2 accumulation in neurons or astrocytes (*18*) and by the inability of PGE_2 to interfere with [³H]anandamide accumulation (Fig. 2A). Previous results indicating that expression of PGE_2 transporter mRNA in brain tissue is not detectable further support this conclusion (*9*).

To search for more potent anandamide transport inhibitors, we synthesized and tested a series of structural analogs of anandamide (*19*). From this screening, we selected the compound *N*-(4-hydroxyphenyl)arachidonylamide (AM404), which was both efficacious and relatively potent (Fig. 3, C and D; IC_{50} was 1 μM in neurons and 5 μM in astrocytes). As we anticipated from its chemical structure, AM404 acted as a competitive inhibitor (*20*), suggesting that it may serve as a transport substrate or pseudosubstrate. In contrast, at the concentrations tested AM404 had no effect on FAAH activity (IC_{50} > 30 μM) or on uptake of [³H]arachidonate or [³H]ethanolamine (102 ± 4% and 96 ± 14%, respectively, at 20 μM AM404, n = 6). Furthermore, a positional isomer of AM404, *N*-(3-hydroxyphenyl)-arachidonylamide (AM403), was significantly less effective than AM404 in inhibiting transport (Fig. 3, C and D). These data provide pharmacological evidence for the existence of a specific anandamide transporter and suggest (i) that neurons and astrocytes may act synergistically in the brain to dispose of extracellular anandamide and (ii) that the transport systems in these two cell types may differ kinetically and pharmacologically (Fig. 1, B and C, and Fig. 3, C and D).

The identification of inhibitors allowed us to examine whether transmembrane transport participates in terminating anandamide responses mediated by cannabinoid receptor activation. Cannabinoid receptors of the CB1 subtype are expressed in neurons (*21*) where they are negatively coupled to adenylyl cyclase activity (*22*). Accordingly, in cultures of rat cortical neurons the cannabinoid receptor agonist WIN-55212-2 inhibited forskolin-stimulated adenosine 3',5'-monophosphate (cAMP) accumulation (control: 39 ± 4 pmol per milligram of protein; 3 μM forskolin: 568 ± 4 pmol per milligram of protein; forskolin plus 1 μM WIN-55212-2: 220 ± 24 pmol per milligram of protein), and this inhibition was prevented by the antagonist SR-141716-A (1 μM) (555 ± 39 pmol/mg of protein, n = 9) (*23*). Anandamide produced a similar effect, but with a potency (IC_{50}, 1 μM) that was 1/20 of that expected from its binding

constant for CB1 cannabinoid receptors (K_i = 50 nM) (1) (Fig. 4A). The transport inhibitor AM404 bound to CB1 receptors with low affinity (K_i = 1.8 μM) (19) and did not reduce cAMP concentrations when applied at 10 μM (Fig. 4B). Nevertheless, the drug enhanced the effects of anandamide, increasing the potency (by a factor of 10) and decreasing the threshold (by a factor of 1/100), an effect that was prevented by SR-141716-A (Fig. 4A). Thus, a concentration of anandamide that was below threshold when applied alone (0.3 μM) produced an almost maximal effect when applied with AM404 (Fig. 4B). Bromcresol green and AM403, which were less effective than AM404 in inhibiting anandamide transport (Fig. 3), were also less effective in enhancing the anandamide response (Fig. 4B). Furthermore, the decreases in cAMP concentrations produced by WIN-55212-2 (which stimulates CB1 receptors but is not subject to physiological clearance) or glutamate [which stimulates metabotropic receptors negatively coupled to adenylyl cyclase (24) and is cleared by a selective transporter (25)] are not affected by any of the anandamide transport inhibitors tested (26).

These results suggest that pharmacological blockade of carrier-mediated transport protects anandamide from physiological inactivation, enhancing the potency of anandamide to nearly that expected from its affinity for CB1 cannabinoid receptors in vitro. To find out whether this potentiation occurs in vivo, we tested the effects of AM404 on the antinociceptive activity of anandamide in mice. Intravenous anandamide (20 mg per kilogram of body weight) elicited a modest but significant analgesia, as measured by the hot plate test (27) ($P < 0.05$, Student's t test), this analgesia disappeared 60 min after injection and was prevented by SR-141716-A (Fig. 4C) (28). Administration of AM404 (10 mg/kg, intravenously) had no antinociceptive effect within 60 min of injection but significantly enhanced and prolonged anandamide-induced analgesia (Fig. 4C) ($P < 0.01$, Student's t test).

Our findings indicate that a high-affinity transport system present in neurons and astrocytes has a role in anandamide inactivation by removing this lipid mediator from the extracellular space and delivering it to intracellular metabolizing enzymes such as FAAH (5, 6). Therefore, the identification of selective inhibitors of anandamide transport should be instrumental in understanding the physiological roles of the endogenous cannabinoid system and may lead to the development of therapeutic agents.

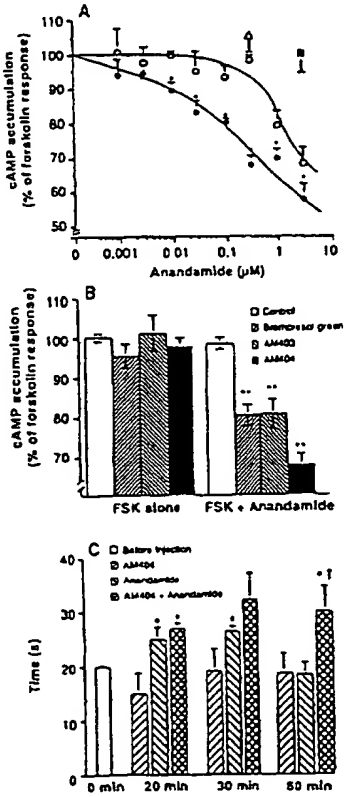


Fig. 4. (A) Effects of AM404 on anandamide-induced inhibition of adenylyl cyclase activity in cortical neurons. The neurons were stimulated with forskolin (3 μM) in the presence of anandamide (0.001 to 3 μM; open circles), anandamide (0.001 to 3 μM) plus AM404 (10 μM) (filled circles), anandamide (3 μM) plus SR-141716-A (1 μM) (square), or anandamide (0.3 μM) plus AM404 (10 μM) and SR-141716-A (1 μM) (triangle). (B) Effects of anandamide transport inhibitors on anandamide-induced inhibition of adenylyl cyclase activity. Forskolin (FSK)-stimulated neurons were incubated with AM404, AM403, or bromcresol green (each at 10 μM) without (FSK alone) or with (FSK + anandamide) 0.3 μM anandamide. Results are expressed as mean ± SEM of nine independent determinations. One asterisk indicates $P < 0.05$ and two asterisks $P < 0.01$ (ANOVA followed by Bonferroni test). (C) Effects of AM404 on the analgesic activity of anandamide in the hot plate test. Three groups of six mice received AM404 (10 mg/kg, intravenous), anandamide (20 mg/kg, intravenous), or anandamide plus AM404. The hot plate test (55.5°C) was performed at the times indicated, and latency to jump (in seconds) was measured before (control) and after the drugs were injected. In all groups, latency to jump before injections was 21 ± 0.6 s (n = 18). A fourth group of mice received injections of vehicle alone (saline containing 20% dimethyl sulfoxide), which did not affect latency to jump. One asterisk indicates $P < 0.05$ compared with uninjected controls (ANOVA followed by Bonferroni test), and one cross indicates $P < 0.01$ compared with anandamide-treated animals (Student's t test).

REFERENCES AND NOTES

1. W. A. Devane *et al.*, *Science* 258, 1946 (1992); R. Mechoulam, L. Hanuš, B. R. Martin, *Biochem. Pharmacol.* 48, 1537 (1994).
2. W. L. Dewey, *Pharmacol. Rev.* 38, 151 (1986).
3. V. Di Marzo *et al.*, *Nature* 372, 686 (1994).
4. H. Cadas, S. Gaillet, M. Beltramo, L. Venance, D. Piomelli, *J. Neurosci.* 16, 3934 (1996); T. Sugura *et al.*, *Eur. J. Biochem.* 240, 53 (1996); H. Cadas, E. di Tomaso, D. Piomelli, *J. Neurosci.*, 17, 1226 (1997).
5. D. G. Deutsch and S. Chin, *Biochem. Pharmacol.* 46, 791 (1993); F. Desarnaud, H. Cadas, D. Piomelli, *J. Biol. Chem.* 270, 6030 (1995); N. Ueda, Y. Kurahashi, S. Yamamoto, T. Tokunaga, *ibid.*, p. 23823.
6. B. Cravatt *et al.*, *Nature* 384, 83 (1996).
7. W. D. Stein, *Channels and Pumps. An Introduction to Membrane Transport*, (Academic Press, San Diego, 1990), pp. 53–57.
8. L. Z. Bito, *Nature* 256, 1234 (1975); J. E. Schaffer and H. F. Lodish, *Cell* 79, 427 (1994); I. N. Bojesen and E. Bojesen, *Acta Physiol. Scand.* 156, 501 (1996).
9. N. Kanai *et al.*, *Science* 268, 866 (1995).
10. Cultures of cortical neurons [N. Sheta, L. Pellerin, P. Magistretti, *J. Neurosci.* 15, 3307 (1995)] or astrocytes (13) were prepared from rat embryos and were used after 4 to 6 days and 21 to 25 days in vitro, respectively. Accumulation of [^{3}H]anandamide (221 Ci/mmol, New England Nuclear, Wilmington, DE) was measured by incubating the cells (six-well plates) for various times in Krebs buffer (136 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, and 20 mM Tris/Hepes base; pH 7.4), at 37°C, containing [^{3}H]anandamide (0.45 nM, brought to 100 nM with nonradioactive anandamide). Incubations were stopped by aspirating the media, and cells were rinsed with Krebs buffer containing bovine serum albumin (BSA, 0.1% w/v) and subjected to extraction with methanol and chloroform. Radioactivity in the extracts was measured directly or after fractionation of cell lipids by thin-layer chromatography (13). For kinetic analyses, the neurons were incubated for 4 min at 37°C in the presence of 10 to 500 nM anandamide containing 0.05 to 2.5 nM [^{3}H]anandamide. We subtracted nonspecific accumulation (measured at 0° to 4°C) before determining kinetic constants by Lineweaver-Burk analysis.
11. M. Beltramo and D. Piomelli, unpublished data.
12. E. L. Barker and R. D. Blakely, in *Psychopharmacology: The Fourth Generation of Progress*, F. E. Bloom and D. J. Kupfer, Eds. (Raven, New York, 1995), pp. 321–334. In a previous study with mixed cultures of rat cortical neurons and astrocytes, a K_m of 30 μM for [^{3}H]anandamide accumulation was obtained (3). Such a high value likely resulted from the low specific radioactivity (0.3 mCi/mmol) of the [^{3}H]anandamide used.
13. M. Beltramo, E. di Tomaso, O. Piomelli *FEBS Lett.* 403, 263 (1997).
14. B. Koutek *et al.*, *J. Biol. Chem.* 269, 22937 (1994).
15. L. Z. Bito, H. Davson, E. V. Salvador, *J. Physiol.* 256, 257 (1976); W. Stremmel and P. D. Berk, *Proc. Natl. Acad. Sci. U.S.A.* 83, 3086 (1986); S. Ruetz and P. Gros, *Cell* 77, 1071 (1994).
16. In astrocytes, V_{max} values for [^{3}H]anandamide accumulation were 200 pmol/min per milligram of protein without bromcresol green, and 111 pmol/min per milligram of protein with bromcresol green (10 μM). Apparent K_m values were 0.24 and 0.25 μM, respectively (n = 6).
17. Displacement of [^{3}H]WIN-55212-2 binding (40 to 60 Ci/mmol; New England Nuclear) to rat cerebellar

membranes (0.1 mg/ml) was determined as described [J. E. Kuster *et al.*, *J. Pharmacol Exp Ther* 264, 1352 (1993)]. Nonspecific binding was measured in the presence of 1 μM nonradioactive WIN-55212-2. FAAH activity was measured in rat brain particulate fractions as described (13). The uptake of [^{3}H]arachidonate (Amersham, 200 Ci/mmol, 5 nM brought to 100 nM) and [^{3}H]ethanolamine (Amersham, 50 Ci/mmol; 20 nM brought to 100 nM) was determined on cortical astrocytes for 4 min as described (10). The control uptake for [^{3}H]arachidonate was 16729 ± 817 dpm per well and for [^{3}H]ethanolamine it was 644 ± 100 dpm per well (n = 6).

18. Neurons or astrocytes were incubated for 4 min at 37°C in Krebs buffer containing [^{3}H]PGE_2 (0.67 nM brought to 100 nM with nonradioactive PGE_2, 171 Ci/mmol, New England Nuclear). After rinsing with Krebs buffer containing BSA, we subjected the cells to lipid extraction and counted radioactivity in the extracts. On average, neurons contained 245 ± 65 dpm per well and astrocytes 302 ± 20 dpm per well; nonspecific accumulation in astrocytes at 0° to 4°C was 355 ± 28 dpm per well (n = 6).
19. A. Khanolkar *et al.*, *J. Med. Chem.* 39, 4515 (1996).
20. In astrocytes, apparent K_m values for [^{3}H]anandamide accumulation were 0.11 μM without AM404 and 0.27 μM with AM404 (10 μM). V_{max} values were 29 pmol/min per milligram of protein without AM404 and 26 pmol/min per milligram of protein with AM404, respectively (n = 6).
21. M. Herkenham *et al.*, *Proc Natl Acad Sci U.S.A.* 87, 1932 (1990); L. A. Matsuda, T. I. Bonner, S. J. Lolait, *J. Comp. Neurol.* 327, 535 (1993).
22. A. C. Howlett, J. M. Qualy, L. L. Khachatrian, *Mol. Pharmacol.* 29, 307 (1986); M. Jung *et al.*, *J. Neurochem.* 68, 402 (1997).
23. Cortical neurons were prepared in 12-well plates and used after 4 to 6 days in vitro. Incubations were carried out in the presence of forskolin (3 μM) and isobutyl methyl xanthine (1 mM). The cAMP concentrations were measured by radioimmunoassay with a commercial kit (Amersham, Arlington, IL) and following manufacturer's instructions.
24. L. Prézeau *et al.*, *Proc Natl Acad Sci U.S.A.* 89, 8040 (1992).
25. R. Malo, M. B. Robinson, M. A. Dichter, *J. Neurosci.* 14, 6754 (1994).
26. The amounts of cAMP in the presence of a concentration of WIN-55212-2 below threshold (1 nM, determined in preliminary experiments) were 96.7 ± 2.5% of forskolin alone and were not significantly affected by 10 μM AM404 (89.8 ± 2.6%), 10 μM AM403 (92.4 ± 2.3%), or 10 μM bromcresol green (92.9 ± 2.3%) (n = 3). In the presence of a concentration of glutamate below threshold (3 μM) (24), cAMP concentrations were 91.6 ± 2% of forskolin alone and were not significantly affected by AM404 (84.4 ± 4.9%), AM403 (89.5 ± 2.4%), or bromcresol green (84.4 ± 3%) (n = 3).
27. E. Fride and R. Mechoulam, *Eur. J. Pharmacol.* 231, 313 (1993); P. B. Smith *et al.*, *J. Pharmacol. Exp. Ther.* 270, 219 (1994).
28. The hot plate test (55.5°C) was carried out on male Swiss mice (25 to 30 g, Nossan, Italy) following standard procedures [F. Porreca, H. L. Mosberg, R. Hurst, V. J. Hruby, T. F. Burks, *J. Pharmacol. Exp. Ther.* 230, 341 (1984)]. Anandamide and AM404 were dissolved in 0.9% NaCl solution containing 20% dimethyl sulfoxide and injected intravenously at 20 mg/kg and 10 mg/kg, respectively. To determine whether cannabinoid receptors participate in the effect of anandamide, we administered anandamide (20 mg/kg intravenously) or anandamide plus SR141716-A (2 mg/kg, subcutaneously) to two groups of six mice each. In mice that received anandamide alone, latency to jump increased from 21.7 ± 1.5 s to 30.7 ± 0.8 s ($P < 0.05$, ANOVA) 20 min after injection. In contrast, in mice that received anandamide plus SR141716-A, the latency to jump was not affected (19.6 ± 3.1 s).
29. We thank E. di Tomaso and H. Cadas for help and E. Barker, L. Parsons, and P. Schweitzer for critical reading of the manuscript. Supported by the Neuroscience Research Foundation, which receives major support from Novartis.

4 February 1997; accepted 17 June 1997